Clinical and Experimental Immunology ORIGINAL ARTICLE doi:10.1111/j.1365-2249.2006.03276.x

Hyperactivity and reactivity of peripheral blood neutrophils in chronic periodontitis

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Summary

Some evidence exists that peripheral neutrophils from patients with chronic periodontitis generate higher levels of reactive oxygen species (ROS) after Fcγ-receptor stimulation than those from healthy controls. We hypothesized that peripheral neutrophils in periodontitis also show both hyper-reactivity to plaque organisms and hyperactivity in terms of baseline, unstimulated generation and release of ROS. Peripheral neutrophils from chronic periodontitis patients and age/sex/smoking-matched healthy controls (18 pairs) were assayed for total ROS generation and extracellular ROS release, with and without stimulation (Fcγ-receptor and *Fusobacterium nucleatum*), using luminol and isoluminol chemiluminescence. Assays were performed with and without priming with *Escherichia coli* lipopolysaccharide (LPS) and granulocyte–macrophage colony-stimulating factor (GM-CSF). Phox gene expression (p22, p47, p67, gp91) was investigated using reverse transcription–polymerase chain reaction (RT–PCR). Neutrophils from patients produced higher mean levels of ROS in all assays. Total generation and extracellular release of ROS by patients' cells were significantly greater than those from controls after FcγR-stimulation, with ($P = 0{\cdot}023$) and without ($P \leq 0{\cdot}023$) priming with GM-CSF. Differences in unstimulated total ROS generation were not significant. By contrast, patients' cells demonstrated greater baseline, extracellular ROS release than those from controls ($P = 0{\cdot}004$). This difference was maintained after priming with LPS ($P = 0{\cdot}028$) but not GM-CSF ($P = 0{\cdot}217$). Phox gene expression was similar in patient and control cells at baseline and stimulation with *F. nucleatum* (3 h) consistently reduced $gp91^{PHOX}$ transcripts. Our data demonstrate that peripheral neutrophils from periodontitis patients exhibit hyper-reactivity following stimulation (Fcγ-receptor and *F. nucleatum*) and hyperactivity in terms of excess ROS release in the absence of exogenous stimulation. This hyperactive/-reactive neutrophil phenotype is not associated with elevated phox gene expression.

Keywords: chronic periodontitis, Fcγ-receptor, *Fusobacterium nucleatum*, GM-CSF, neutrophil hyperactivity, phox gene expression

Accepted for publication 3 November 2006
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Introduction

A number of major pathological conditions are mediated by neutrophil-derived oxidant injury, including rheumatoid arthritis, diabetes, myocardial infarction, stroke and inflammatory lung disease [1,2]. Furthermore, recent studies of a relatively uncommon form of periodontal disease, localized aggressive periodontitis, suggest that peripheral neutrophils are hyper-responsive in respect of reactive oxygen species (ROS) generation after exposure to leukotriene B4 (LTB4) and interleukin (IL)-8 due to reduced gene and protein expression of diacylglycerol (DAG) kinase [3], inhibition of which is known to amplify the respiratory burst in normal neutrophils [4].

Chronic periodontitis is a major cause of tooth loss in the developed world and is now recognized as a significant risk

factor for cerebrovascular disease (stroke), type II diabetes and cardiovascular disease, independently of smoking status [5]. Current evidence indicates that chronic periodontitis occurs in predisposed individuals who have an abnormal inflammatory/immune response to specific organisms within the microbial plaque biofilm which accumulates at the gingival margin [6,7]. Although the abnormal response to plaque appears to involve the excessive generation of ROS [8] and release of proteases [9] by neutrophils *in vitro*, the demonstration of altered ROS generation by peripheral neutrophils in chronic periodontitis is highly technique-dependent (reviewed by Chapple & Matthews [8]). However, using a luminol detection system for total ROS production in the absence of divalent cations, data from a Swedish group have demonstrated consistently a small but significantly higher level of ROS generation by FcγR-stimulated peripheral neutrophils isolated from chronic periodontitis patients compared to age- and gender-matched periodontally healthy controls [10–13]. Interestingly, although only very low FcγR-stimulated ROS could be detected in the extracellular compartment using isoluminol as a substrate, a similar difference between patients and controls was found [13]. The underlying basis for this hyper-reactive neutrophil phenotype in chronic periodontitis is unclear and does not appear to be associated with reduced expression of DAG kinase [3], expression of adhesion molecules [10], FcγR polymorphisms [13,14] or the method of neutrophil preparation [12], or *in vitro* priming with tumour necrosis factor (TNF)-α, *Escherichia coli* lipopolysaccharide (LPS), fMetLeuPhe or ArgGlyAspSer [11,15].

Granulocyte–macrophage colony-stimulating factor (GM-CSF) and the presence of *Fusobacterium nucleatum* are two potential factors that may be involved in both local and peripheral priming and/or stimulation of neutrophils in chronic periodontitis which have not been investigated. GM-CSF is known to be up-regulated in neutrophil-mediated pathology [16,17] and is associated with periodontal inflammation in some patients after GM-CSF therapy [18]. It has a variety of effects on neutrophils potentially important in the pathogenesis of periodontitis, including dose-dependent chemotaxis or inhibition of movement, inhibition of apoptosis and priming for increased phagocytic and respiratory burst activity [19–21]. Similarly, *F. nucleatum* is a key quorum-sensing organism present in subgingival plaque [22] and associated with chronic periodontitis, which can induce proinflammatory cytokine (IL-1β, TNF-α, IL-8), elastase and ROS production by peripheral neutrophils [23,24].

To date, investigations of baseline, unstimulated ROS generation by peripheral neutrophils in chronic periodontitis have found no differences between patient and control cells [25–27]. Although luminol-dependent chemiluminescence from unstimulated neutrophils in the absence of divalent cations is reported to be negligible, addition of Ca^{2+} and Mg^{2+} significantly increases the chemiluminescent signal [11,13,28]. Unfortunately, there are no studies of unstimulated ROS generation in periodontitis using luminol or isoluminol in the presence of Mg^{2+} and Ca^{2+}.

Nicotinamide adenine dinucleotide phosphate (NADPH) oxidase is crucial to the production of ROS by activated neutrophils and is a highly regulated enzyme complex composed of cytosolic (e.g. $p40^{PHOX}$, $p47^{PHOX}$, $p67^{PHOX}$) and membrane-bound (e.g. $p22^{PHOX}$, $gp91^{PHOX}$) proteins. When activation occurs, the cytosolic components translocate to the membrane, associate with the other components and form the active oxidase which catalyses the production of superoxide. Superoxide is short-lived, dismutates to hydrogen peroxide and forms other secondary ROS [29]. To date, there have been no studies to investigate whether the expression of genes coding for NADPH oxidase components are altered in neutrophils from chronic periodontitis patients.

The purpose of this study was (i) to confirm the reported FcγR hyper-reactivity of peripheral neutrophils in chronic periodontitis using more relevant physiological conditions (i.e. in the presence of divalent cations) and (ii) because of the greater activity of cells under these conditions [11,13,28] to determine whether differences in baseline, unstimulated generation of ROS between periodontal health and disease could be detected. Having established these differences, additional studies were performed to determine neutrophil responsiveness to *F. nucleatum* and the effect of priming with GM-CSF on FcγR-stimulated ROS production by patient and control cells. Finally, preliminary gene expression studies were performed to determine whether phox transcripts were differentially expressed in health and disease, as such differences have been reported in type II diabetes, a known risk factor for chronic periodontitis [30,31].

Materials and methods

Patients

Subjects with chronic periodontitis ($n = 18$; five males and 13 females; mean age, 47·2 ± 6·1 years, range, 36–61 years) were recruited from patients referred to the periodontal department at Birmingham Dental Hospital. Chronic periodontitis was defined as described previously [32]. Age- and sex-matched periodontally healthy control subjects ($n = 18$; five males and 13 females; mean age, 46·4 ± 5·4 years, range, 37–56 years) were recruited from staff of the Dental Hospital. All subjects were systemically healthy. Exclusion criteria included pregnancy, use of non-steroidal anti-inflammatory or anti-microbial drugs; and mouthwashes or vitamin supplements within the previous 3 months. All volunteers were never smokers, did not use recreational drugs and had no special dietary requirements. Ethical approval was granted by South Birmingham Local Research Ethics Committee (LREC 5643). After providing informed consent, subjects completed a medical questionnaire.

Collection of venous blood and preparation of neutrophils

Venous blood was collected into Vacutainer™ (Greiner, Bio-One Ltd, Stonehouse; UK) lithium heparin (17 IU/ml) tubes from a patient and paired age/gender-matched control between 09·00 h and 10·00 h in the morning following an overnight fast. Compliance with abstention was checked prior to sampling. Neutrophils were isolated using a discontinuous Percoll gradient ($\delta = 1{\cdot}079 : 1{\cdot}098$; 8 min at 150 ***g***, 10 min at 1200 ***g***) followed by erythrocyte lysis [0·83% NH_4Cl containing 1% $KHCO_3$, 0·04% Na_2 ethylenediamine tetraacetic acid (EDTA) $2H_2O$ and 0·25% bovine serum albumin (BSA) for 20 min]. Isolated cells were washed and resuspended in phosphate-buffered saline (PBS) supplemented with glucose (1 mM) and cations (1 mM $MgCl_2$, 1·5 mM $CaCl_2$) at 1×10^6 cells/ml. Cell viability, typically > 98%, was determined immediately prior to assay using trypan blue exclusion.

Bacterial culture and suspensions

Staphylococcus aureus (NCTC 6571) was grown in air on mannitol salt agar and inoculated into tryptone soy broth. The periodontopathogens *F. nucleatum* (ATCC 10953) and *Porphyromonas gingivalis* (ATCC 33277) were grown anaerobically at 37°C essentially as described previously [33]. Bacteria were isolated from broth cultures by centrifugation, washed three times in sterile PBS, heat-treated (100°C for 10 min) prior to dilution with sterile PBS to give a final suspension of 4×10^8 cells/ml which was stored at −30°C.

Opsonized *S. aureus* was prepared as described by Bergström and Åsman [34] and stored as a $1{\cdot}2 \times 10^9$ cells/ml suspension at −80°C.

Enhanced chemiluminescent assay

Chemiluminescence assays were performed using luminol to detect total radical generation (intra- and extracellular) and isoluminol, to detect extracellular radical production. All assays were performed (37°C) using a Berthold microplate-luminometer (LB96v). Thirty-five µl of supplemented PBS, 30 µl luminol (3 mmol/l) or 60 µl isoluminol (3 mmol/l) with 6 U horseradish peroxidase were added to preblocked (PBS containing 1% BSA, overnight, 4°C) white microwells (Microlite 2, VWR, Lutterworth, UK). The plate was then placed into the microplate reader and 100 µl of buffer containing the isolated neutrophils (1×10^5 cells) were then added to each well and incubated for 30 min at 37°C. Cells were then stimulated with 25 µl of opsonized *S. aureus* (300 bacteria/neutrophil), *F. nucleatum* suspension (100 bacteria/neutrophil) or PBS (unstimulated control). All samples were run in triplicate, with paired patient and control samples analysed at the same time. Light emission in relative light units (RLUs) was recorded during the 30-min prestimulation period to study baseline, unstimulated radical release and after stimulation for 150 min. Peak RLUs were determined for both pre- and post-stimulation incubation periods.

The same protocol was used to study the effect of neutrophil priming except that 10 µl of *E. coli* LPS (5 µg/ml, equivalent to 50 ng/10^5 cells), GM-CSF (1·25 ng/ml, equivalent to 12·5 pg/10^5 cells) or PBS (unprimed) were added to the wells immediately after the cells prior to the initial 30-min incubation/priming period. LPS was included, as it has been studied previously in this assay system [11,15].

Stimulation of neutrophils for gene expression analyses

Neutrophils (1×10^6 cells) in 1 ml of PBS were added to 500 µl of buffer containing either opsonized *S. aureus* (3×10^8 bacteria), *F. nucleatum* (1×10^8 bacteria), *P. gingivalis* (1×10^8 bacteria) or *E. coli* LPS (0·1 µg and 1 µg) and mixed gently prior to incubation (uncapped) at 37°C for 3 h.

Neutrophil RNA isolation and cDNA synthesis

Following incubation neutrophils were pelleted, the supernatant removed, 1 ml of Trizol (Invitrogen, Paisley, UK) added and the cell pellet resuspended by vigorous mixing. Following phenol/chloroform extraction (Sigma, Gillingham, UK) the aqueous phase was combined with 70% ethanol and loaded onto an RNeasy mini-column (Qiagen, Crawley, UK). Subsequent purification and DNase treatment were performed as recommended by the manufacturer (Qiagen). RNA was eluted in 30 µl of sterile water.

For cDNA synthesis, 1–2 µg of DNase digested total RNA was incubated with oligo(dT) (Ambion, Huntingdon, UK) for 1 h at 37°C for reverse transcription to generate single-stranded cDNA using the Omniscript kit (Qiagen). Both RNA and cDNA concentrations were determined using a Biophotometer (Helena Biosciences, Sunderland, UK).

Semi-quantitative reverse transcription–polymerase chain reaction (RT–PCR)

Semi-quantitative RT–PCR (sq-RT–PCR) for $p22^{PHOX}$, $gp91^{PHOX}$, $p47^{PHOX}$ and $p67^{PHOX}$ was performed on unstimulated and *F. nucleatum*-stimulated neutrophils from patients ($n = 5$) and their corresponding matched controls ($n = 5$). In addition, sq-RT–PCR was also performed using pooled RNA isolated from neutrophils from healthy controls ($n = 5$) with and without stimulation with *E. coli* LPS, *P. gingivalis* and opsonized *S. aureus*. Typically, 50 ng of cDNA were used to seed 25 µl RED*Taq* PCR mixes (Sigma, Gillingham,UK) containing 1 µl 25 mM forward and reverse primer and subjected to between 27 and 37 cycles. Primer sequences and cycling conditions used are shown in Table 1. Following denaturation at 95°C for 5 min, a typical amplification cycle of 95°C for 20 s, 60/61°C for 20 s and 72°C for 20 s was performed using a Mastercycler thermal cycler (Helena Biosciences, Sunderland, UK). Following the designated number of cycles, 7 µl of the PCR mix was removed and the product

Table 1. Primer sequences and conditions used for semiquantitative reverse transcription–polymerase chain reaction analysis.

Gene†	Primer sequence (5′–3′)	T_m	Product	Cycle number
GP91*phox*	(f)/GCT GTT CAA TGC TTG TGG CT (r)/TCT CCT CAT CAT GGT GCA CA	61	403 bp	30
P22*phox*	(f)/GTT TGT GTG CCT GCT GGA GT (r)/TGG GCG GCT GCT TGA TGG T	60	325 bp	27
P67*phox*	(f)/CGA GGG AAC CAG CTG ATA GA (r)/CAT GGG AAC ACT GAG CTT CA	60	726 bp	37
P47*phox*	(f)/ACC CAG CCA GCA CTA TGT GT (r)/AGT AGC CTG TGA CGT CGT CT	60	767 bp	30
GAPDH	(f)/CCA CCC ATG GCA AAT TCC ATG GCA (r)/TCT AGA CGG CAG GTC AGG TCC ACC	60	391 bp	27

T_m: annealing temperature (°C); (f) = forward primer; (r) = reverse primer. †Primer sequences for Phox and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) gene expression were taken from Jones *et al.* [35] and McLachlan *et al.* [36], respectively.

separated and visualized on a 1·5% agarose gel containing 0·5 μg/ml ethidium bromide (Sigma). Following UV illumination gel images were captured using electrophoresis documentation and analysis system (EDAS) 120 software (Kodak, New Haven, USA). Automatic interpretation and data analysis software (AIDA) (Raytest, Sheffield, UK) was used to determine the volume density of amplified products, which were subsequently normalized against glyceraldehyde-3-phosphate dehydrogenase (GAPDH) control values.

Data handling and statistical analysis

Chemiluminescent data were recorded automatically into Microsoft Excel. Data were manipulated in Excel and statistical evaluation performed using Minitab (version 14). The Wilcoxon rank sum test was used for all statistical comparisons except for patient *versus* control differences in Phox gene expression, which was performed using the Mann–Whitney *U*-test. Detection of ROS by chemiluminescence methods can show considerable day-to-day variation making the inclusion of a paired, age and gender matched control, whose neutrophils are analysed simultaneously with those from the patient, important if consistent and comparable results are to be obtained [37]. Data derived from such experiments are thus normally analysed using the Wilcoxon (one-tailed) test to determine the presence of neutrophil hyper-reactivity [11–13]. The one-tailed Wilcoxon test was also used to compare Phox gene expression in unstimulated and *F. nucleatum*-stimulated cells based on the preliminary experiments using pooled RNA of neutrophils from periodontally healthy individuals. A level of $P < 0{\cdot}05$ was employed for assigning statistical significance.

Results

ROS generation by unstimulated neutrophils and after FcγR stimulation

Isolated, unstimulated neutrophils demonstrated measurable levels of both total and extracellular chemiluminescence during the 30-min assay period (Fig. 1). Although the kinetics of light output were similar for luminol and isoluminol, with peak light signals being obtained at 15·32 ± 3·49 min and 16·32 ± 3·42 min, respectively, the amount of chemiluminescence was significantly greater with

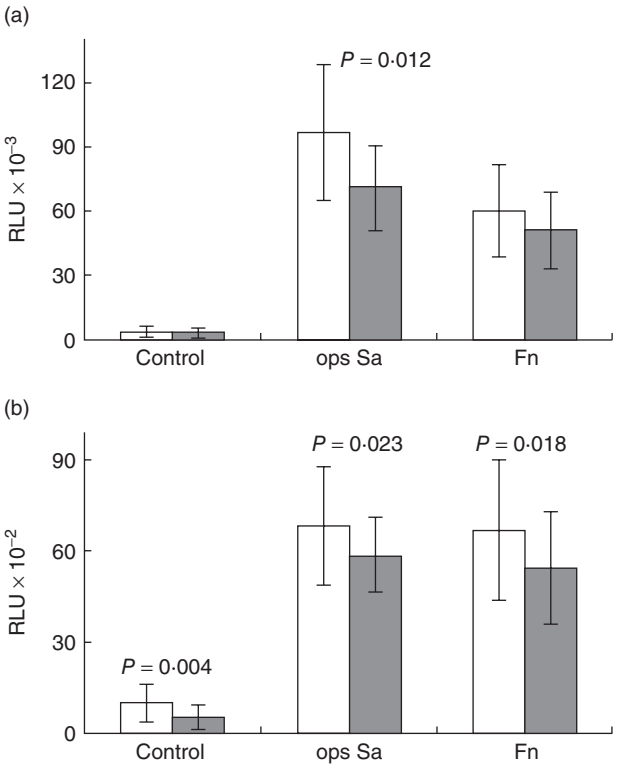

Fig. 1. Mean (± s.d.) chemiluminescence (a, luminol-dependent, total chemiluminescence; b, isoluminol-dependent, extracellular chemiluminescence) generated by peripheral neutrophils isolated from patients (□; $n = 18$) and periodontally healthy individuals (■; $n = 18$) in the absence of stimulant (control) or stimulated with opsonized *Staphylococcus aureus* (ops Sa) or unopsonized *Fusobacterium nucleatum* (Fn). *P*-values calculated using one-tailed Wilcoxon test. Unopsonized *S. aureus* did not elicit a detectable chemiluminescent signal.

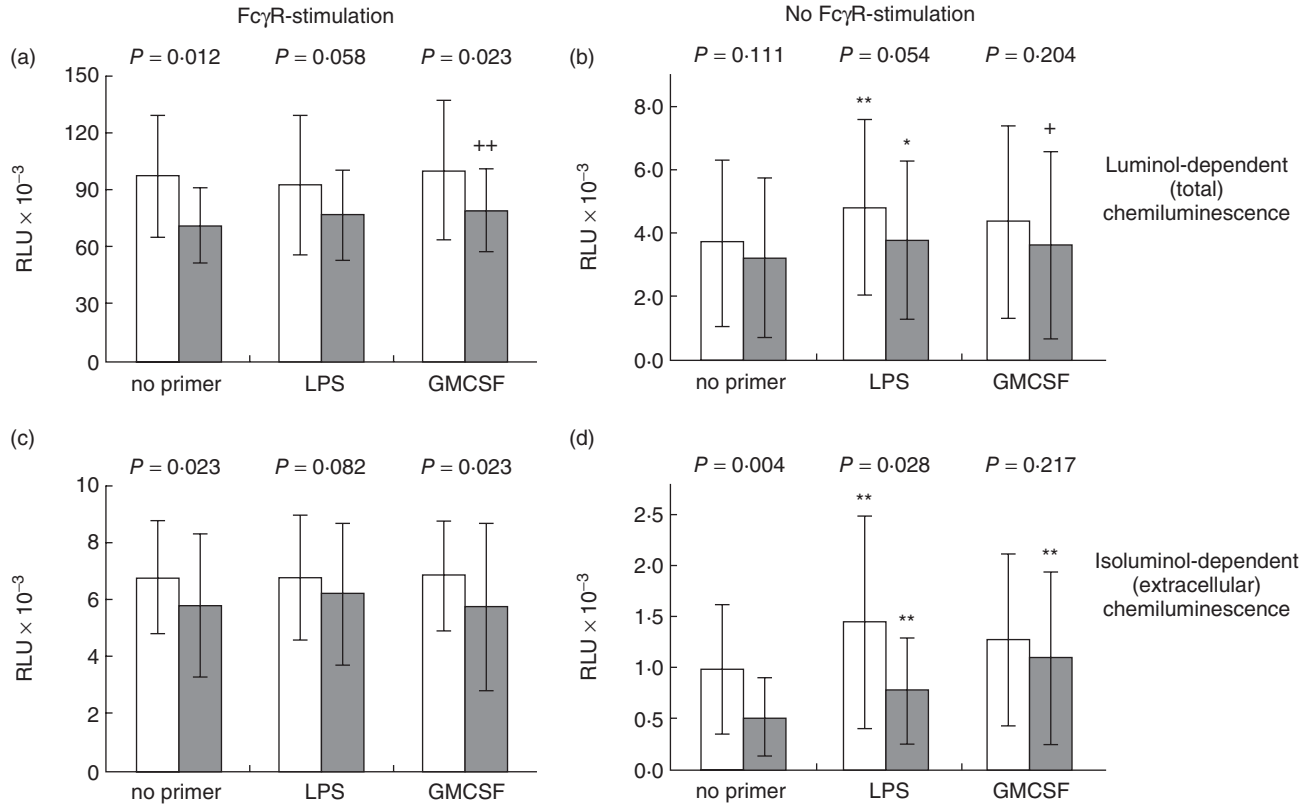

Fig. 2. Mean chemiluminescence (relative light unit $\times 10^{-3}$; $\pm$ s. d.) generated by peripheral neutrophils isolated from patients (□; $n = 18$) and matched periodontally healthy individuals (■; $n = 18$). Chemiluminescence was monitored after incubation of cells with priming agent (lipopolysaccharide or granulocyte–macrophage colony-stimulating factor) or phosphate-buffered saline (no primer control) with (a, c) and without (b, d) subsequent FcγR stimulation with opsonized *Staphylococcus aureus*. Significant differences between primed and unprimed cells ($^{+}P = 0{\cdot}041$; $^{++}P = 0{\cdot}008$; $^{*}P = 0{\cdot}001$; $^{**}P < 0{\cdot}0001$) and between patient and control cells (P-values) were calculated using one-tailed Wilcoxon test. Unopsonized *S. aureus* did not elicit a detectable chemiluminescent signal.

luminol ($P < 0{\cdot}0001$). Unstimulated neutrophils from patients and controls did not differ significantly in terms of total chemiluminescence ($P = 0{\cdot}11$; Fig. 1a) but cells from patients exhibited greater extracellular light output than those from controls ($P = 0{\cdot}004$; Fig. 1b). Stimulation with opsonized *S. aureus* significantly increased both total ($P < 0{\cdot}0001$) and extracellular ($P < 0{\cdot}0001$) light output from baseline levels. Furthermore, neutrophils from periodontitis patients generated significantly higher levels of total ($P = 0{\cdot}012$) and extracellular ($P = 0{\cdot}023$) chemiluminescence than those from controls (Fig. 1).

ROS generation after stimulation with *F. nucleatum*

Stimulation of both patient and control neutrophils with *F. nucleatum* induced significant production of both total and extracellular chemiluminescence ($P < 0{\cdot}0001$) compared to baseline levels (Fig. 1). The level of luminol-dependent chemiluminescence induced by *F. nucleatum* was lower than that obtained with opsonized *S. aureus* for both patient ($P = 0{\cdot}0007$) and control ($P = 0{\cdot}002$) cells. Although patient cells produced higher mean and median levels of light output compared to those from controls, the difference was not statistically significant ($P = 0{\cdot}096$). The level of isoluminol chemiluminescence generated by *F. nucleatum* was equivalent to that obtained using opsonized *S. aureus* and patients' neutrophils generated significantly higher light output than those from controls ($P = 0{\cdot}018$).

Priming and stimulatory effects of *E. coli* LPS and GM-CSF

Generally, priming neutrophils with LPS or GM-CSF prior to FcγR stimulation had no significant effect on the levels of chemiluminescence generated, with both mean and median levels of light output always being greatest for patients' cells (Fig. 2a,c). The exception to this was a small but significant increase in luminol-dependent total chemiluminescence by control cells after GM-CSF treatment ($P = 0{\cdot}008$). Despite this, neutrophils from patients generated significantly higher levels of chemiluminescence than controls ($P = 0{\cdot}023$).

Treatment of both patient and control cells with LPS, without subsequent stimulation with opsonized *S. aureus*, significantly increased total and extracellular chemilumines-

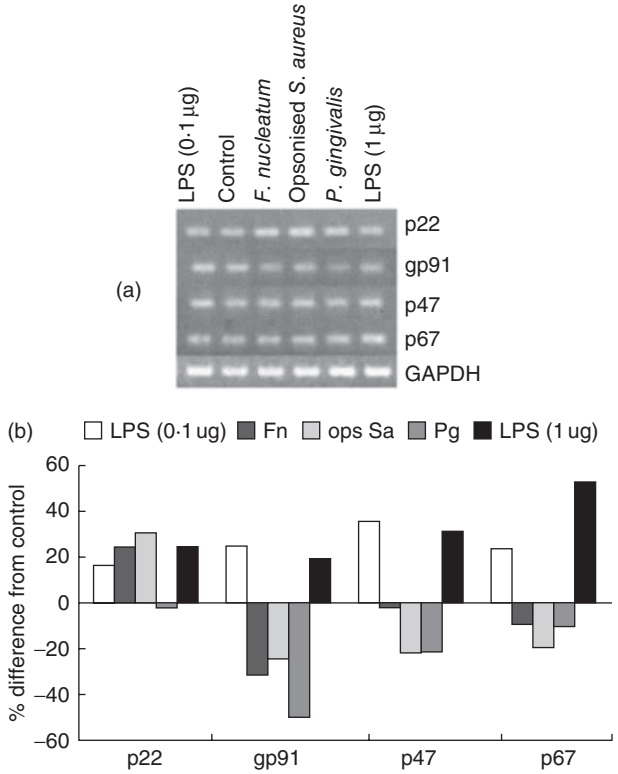

Fig. 3. (a) Gel image of phox gene expression in unstimulated neutrophils (control) and those stimulated via FcγR (opsonized *Staphylococcus aureus*) and with *Fusobacterium nucleatum* (Fn), *P. gingivalis* (Pg) and *Escherichia coli* lipopolysaccharide. (b) Phox gene expression (Phox gene : glyceraldehyde-3-phosphate dehydrogenase ratios) in stimulated neutrophils as a percentage of those in unstimulated, control cells. Data derived from analysis of pooled RNA from individually stimulated cells from five periodontally healthy individuals.

cence compared with control, unprimed cells ($P \leq 0{\cdot}001$; Fig. 2b,d). This effect of LPS did not remove the detectable hyperactivity of patient neutrophils in respect of extracellular radical release (Fig. 2d).

Priming with GM-CSF appeared to affect cells differentially from healthy controls, inducing significantly greater generation of both luminol- and isoluminol-dependent chemiluminescence compared to non-primed cells (luminol, $P = 0{\cdot}041$; isoluminol, $P = 0{\cdot}0001$). Thus, in contrast to LPS, GM-CSF priming resulted in loss of the hyperactive phenotype with the levels of extracellular radical release being similar for patient and control cells (Fig. 2d).

Phox gene expression

Phox gene expression was investigated initially using pooled RNA from peripheral neutrophils of periodontally healthy controls that had been individually stimulated with *F. nucleatum*, *P. gingivalis*, opsonized *S. aureus* and *E. coli* LPS (Fig. 3). The gene expression pattern was altered by stimulation with *E. coli* LPS giving a general increase in transcripts of all of the four phox genes studied. By contrast, stimulation via FcγR or with whole *F. nucleatum* and *P. gingivalis* gave broadly similar expression patterns that were characterized by reduced expression of gp91^{PHOX} and p67^{PHOX}. The major difference between these stimulants was that *P. gingivalis* and *F. nucleatum* had no effect on p22^{PHOX} and p47^{PHOX} expression, respectively.

The effect of stimulation using *F. nucleatum* on phox gene expression was investigated further in five patients and their matched controls (Fig. 4; patient : control pairs 6, 8, 11, 12 and 13). There were no statistically significant differences in phox gene expression between patient and controls. *F. nucleatum* stimulation induced similar changes in phox gene expression in patients and controls, the data being consistent with those obtained with pooled RNA from the original five controls (Fig. 3). The most notable effect of *F. nucleatum* was a significant decrease in gp91^{PHOX} gene expression ($P < 0{\cdot}03$ for both patients and controls).

Discussion

The data demonstrate for the first time that peripheral neutrophils from patients with chronic periodontitis exhibit both hyper-reactivity, after stimulation with *F. nucleatum* or via Fcγ-receptors, and hyperactivity, in the absence of exogenous stimulation. Studies of ROS generation by peripheral blood neutrophils in periodontal disease have used a variety of patient groups, different pathways of activation and methods of ROS detection [8]. It is therefore not surprising that overall there has been no agreement as to whether ROS generation is altered in periodontitis, despite evidence that biomarkers of oxidative stress are increased in the periodontal tissues of periodontitis patients [38–41], and anti-oxidant defences are reduced relative to healthy controls [32]. The most consistent evidence for neutrophil hyper-reactivity in chronic periodontitis has come from a series of studies from a single research group in Sweden showing higher levels of luminol-dependent chemiluminescence generation by Fcγ-receptor-stimulated peripheral neutrophils isolated from chronic periodontitis patients compared to age- and gender-matched controls [10–13,15]. Thus, our data on the effects of FcγR stimulation are the first independent confirmation of these findings.

A novel finding is the hyperactivity of patients' neutrophils in terms of low-level extracellular release of ROS without exogenous stimulation. Such heightened baseline activity has clear implications for the pathogenesis of chronic periodontitis and suggests that either increased ROS release *per se*, or factors increasing neutrophil numbers, life span or the time they remain within the periodontal tissues may be important in oxidative stress-mediated tissue damage. While such damage might be small and biologically/clinically insignificant on its own, it could be crucial to the initiation or progression of disease when combined with the

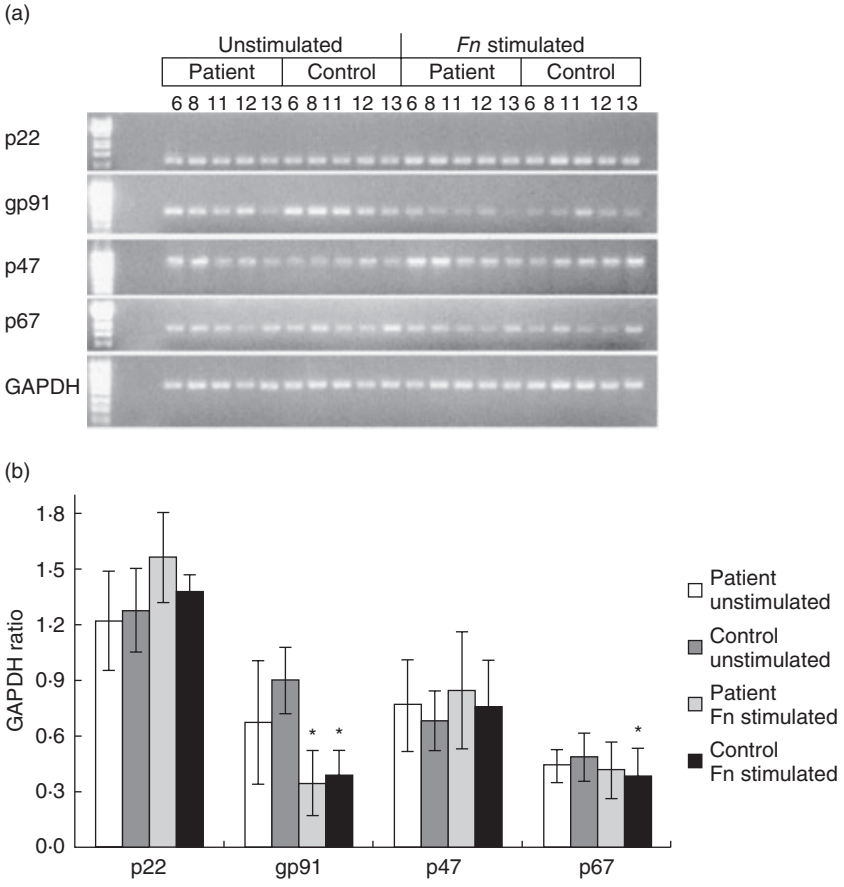

Fig. 4. (a) Gel image of phox gene expression and (b) mean (± s.d.) Phox gene : glyceraldehyde-3-phosphate dehydrogenase ratios derived from unstimulated and *Fusobacterium nucleatum*-stimulated peripheral neutrophils from patients ($n = 5$) and matched controls ($n = 5$). There were no significant differences between patients and controls (Mann–Whitney test). Significant differences between stimulated and unstimulated cells were calculated using Wilcoxon's test (*$P = 0{\cdot}03$, one-tailed).

local stimulatory effects generated, directly or indirectly, by plaque bacteria. Interestingly, recent studies have demonstrated that enhanced baseline respiratory burst activity (intracellular) is a feature of peripheral neutrophils in chronic obstructive pulmonary disease [2]. The lack of any difference in total, luminol-dependent ROS generation by neutrophils in the absence of stimulant is consistent with previous studies that have used either whole blood [26,27] or chemiluminescence assays performed in the absence of divalent cations which significantly reduces the responses of neutrophils [11,13,28].

The finding that peripheral neutrophils from chronic periodontitis patients also exhibit hyper-reactivity to *F. nucleatum* is novel. *F. nucleatum* is a key periodontal pathogen that has been shown, in the absence of serum factors, to induce levels of ROS formation by peripheral neutrophils from healthy donors [42] capable of causing lipid peroxidation [24] and oxidative damage to themselves and surrounding cells [43]. Furthermore, phagocytosis of *F. nucleatum* by neutrophils from healthy individuals induces significantly greater ROS generation than phagocytosis of other periodontal pathogens such as *P. gingivalis* or *Actinobacillus actinomycetemcomitans* [43]. In chronic periodontitis, *F. nucleatum* is normally present within subgingival plaque and associated closely with periodontal tissues containing large numbers of neutrophils that have migrated from blood. The presence of neutrophils hyper-reactive to *F. nucleatum*, especially in terms of stimulating extracellular ROS release, could lead to host tissue damage and the initiation and/or progression of periodontitis.

An important question is whether the hyperactive and -reactive neutrophil phenotype in chronic periodontitis represents a constitutional risk factor for developing the disease or is a result of peripheral priming due to microbial products (e.g. LPS) or the products of the host response to infection (e.g. GM-CSF). Early data investigating Fcγ-receptor-stimulated ROS generation in patients with juvenile periodontitis indicated that hyper-reactivity was present after treatment, supporting the presence of a constitutional defect in this aggressive variant of the disease [44]. More recently, data on neutrophils from patients with chronic periodontitis after successful treatment (i.e. periodontally healthy) have been published, further suggesting that Fcγ-receptor stimulated ROS hyper-responsiveness is constitutional rather than due to *in vivo* priming as a consequence of the disease [13]. Clearly, longitudinal studies investigating ROS-responses of neutrophils before and after treatment are required to determine whether the hyperactivity and -reactivity of peripheral neutrophils in chronic periodontitis is constitutional or the result of peripheral priming due to the disease process.

Published studies indicate that the Fcγ-receptor hyper-reactive neutrophil phenotype in chronic periodontitis is not affected by *in vitro* priming with a variety of agents (TNF-α, *E. coli* LPS, *f*MetLeuPhe, Arg-Gly-Asp-Ser peptide) [11,15]. Our data using *E. coli* LPS to prime cells (0·05 μg/10^5 cells) prior to Fcγ-receptor stimulation confirm the published findings using high-dose LPS (2–8 μg/10^5 cell in the absence of divalent cations), indicating that the FcγR hyper-reactive phenotype is retained after LPS priming [11,15]. However, we also found that low-dose LPS alone caused significant generation of both total and extracellular chemiluminescence and that neutrophils from patients exhibited hyper-reactivity compared to those from matched controls. This was not detected in the previous studies, where the chemiluminescence response of neutrophils to relatively high doses of LPS was reported as negligible. This is not surprising as Ca^{2+} signalling, involving release from intracellular stores and influx from the extracellular fluid, is known to be important in FcγR stimulation and priming of neutrophils for an enhanced respiratory burst [45]. Overall, these findings suggest that neutrophil hyperactivity, with respect to unstimulated, extracellular ROS release, and FcγR hyper-reactivity, in terms of total ROS generation, are not due to peripheral priming by LPS.

In contrast to the LPS results, treatment of neutrophils with GM-CSF had a greater effect on control cells resulting in loss of the hyperactivity of patients' neutrophils in terms of low-level release of extracellular ROS but retention of the Fcγ-receptor hyper-reactive phenotype. Thus, it is possible that GM-CSF could be one factor priming peripheral neutrophils in chronic periodontitis, especially for production of the high baseline levels of extracellular ROS we have detected *in vitro*. Such a role is consistent with the known up-regulation of GM-CSF in neutrophil-mediated pathology [16,17] and observed periodontal inflammation in some patients following GM-CSF therapy [18].

GM-CSF can be produced by many cell types, including epithelium, and up-regulation of gene expression and protein by gingival epithelial cells can be induced by purified components of *P. gingivalis* and *P. intermedia* [46]. Furthermore, we have shown that GM-CSF gene expression is rapidly up-regulated in an oral epithelial cell line (H400 cells) [47] after treatment with whole dead periodontopathogens such as *F. nucleatum* and *P. gingivalis* (unpublished observations). A recent study of GM-CSF in gingival crevicular fluid indicated that the growth factor is detected more commonly at sites associated with periodontitis (63·3%) compared to healthy sites (43·7%) [48]. Thus plaque build-up adjacent to junctional and crevicular epithelium may induce GM-CSF expression that could not only increase baseline radical release from neutrophils in the adjacent tissues but also facilitate recruitment from the blood, immobilization in the periodontal tissues, inhibition of apoptosis, as well as priming for increased phagocytosis and an increased respiratory burst [19–21].

Our data on phox gene expression did not detect any differences between patient and control neutrophils and do not aid our understanding of the neutrophil hyperactivity associated with chronic periodontitis. Further studies are needed in light of the recent data indicating differences in leucocyte $p22^{PHOX}$ gene expression in type II diabetes [30,31], a known risk factor for chronic periodontitis. While the up-regulation of phox genes by *E. coli* LPS is in agreement with published data on granulocytes and monocytic cell lines [49,50], there appear to be no studies on neutrophil phox gene expression after FcγR stimulation or challenge with periodontal pathogens. The most consistent finding after challenging neutrophils from periodontally healthy individuals was a down-regulation of $gp91^{PHOX}$, which was also found after challenging patients' neutrophils with *F. nucleatum*. Interestingly, human granulocytic *Ehrlichiosis* bacteria can completely suppress $gp91^{PHOX}$ gene expression and the respiratory burst response of HL-60 cells to PMA [51], even though PMA normally up-regulates phox gene expression, including $gp91^{PHOX}$ [52]. It is possible that the reduction in $gp91^{PHOX}$ expression detected in our experiments after chronic exposure (3 h) to periodontopathogens and opsonized bacteria could cause a reduction of respiratory burst activity, which may reflect a mechanism protecting cells from prolonged ROS generation. Thus, our findings could suggest that neutrophils within periodontal tissues that are chronically exposed to plaque might exhibit an initial ROS generating and tissue-damaging phase followed by decreased activity whereby periodontal pathogens might not be efficiently killed. Both phases would tend to lead to initiation and development of periodontitis.

In conclusion, we have demonstrated that peripheral blood neutrophils from chronic periodontitis patients display hyper-reactivity when challenged with *F. nucleatum* or via Fcγ-receptor stimulation and hyperactivity with respect to extracellular ROS release even when unprimed and unstimulated. Our data, when interpreted alongside the previous literature, suggest that the hyper-reactivity following Fcγ-receptor stimulation is not due to LPS- or GM-CSF priming of neutrophils. Similarly, the propensity of neutrophils from periodontitis patients to release excess ROS is not due to LPS priming but may, in part, be the result of priming within the peripheral circulation by GM-CSF. These data do not exclude the possible participation of other inflammatory mediators released by the host or plaque flora, or indeed that the cells are inherently more active. A clearer understanding of the latter requires longitudinal studies of neutrophils isolated before and after successful periodontal therapy.

Acknowledgements

This work was supported by the Medical Research Council (grant number: MRC UK-G0000797).

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